RELATION BETWEEN ANAEROBIC ATP SYNTHESIS FROM PYRUVATE AND NITROGEN FIXATION IN *AZOTOBACTER VINELANDII*

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1. Introduction

It is known that the growth of many aerobic and facultatively anaerobic bacteria is inhibited at high oxygen tension [1]. Schmidt-Lorenz [2] showed that in the case of Azotobacter high pO_2 only affected nitrogen fixation and not assimulation of bound nitrogen. In addition Dalton and Postgate [3] demonstrated that nitrogen fixation is an anaerobic process, but the question how the energy, necessary for nitrogen fixation, under these conditions is provided, has not been answered yet.

Anaerobic or facultative anaerobic grown species can use pyruvate as electron and energy donor for nitrogen fixation [4, 5]. This paper reports on the anaerobic ATP synthesis from pyruvate in *Azotobacter vinelandii* and the significance of this process in nitrogen fixation.

2. Materials and methods

NAD^+, NADH, TPP, acetyl-phosphate, ADP, ATP were obtained from the Sigma Chemical Co. Citrate-synthase, hexokinase, glucose-6-phosphate dehydrogenase, CoA, creatine-phosphate and creatine-phosphokinase were obtained from Boehringer.

A. vinelandii OP was grown on a nitrogen-free medium according to Pandit-Hovenkamp [6]. Cells were grown for 24 hr on a rotatory shaker in 5 l flasks (final vol 1 l) under air and harvested each day. After washing with 50 mM Tris-HCl pH 7.5 (1 part cell suspension + 100 parts Tris-HCl), the cells were disrupted anaerobically with a MSE sonic disintegrator (60 W, 60 KHz) at 0° (4 × 30 sec). After centrifugation at 20,000 *g* for 15 min the supernatant was taken (crude extract). All incubations were carried out at 30° in flasks sealed with "Suba seal" closures and gassed with argon (Loos and Co.). When required pure oxygen was added with a syringe through the seal.

The standard incubation mixture contained: 50 mM pyruvate, 0.1 mg/ml CoA, 2 mM DTT, 0.5 mM TPP, 0.5 mM NAD^+, 2 mM EDTA, 4 mM $MgCl_2$, 2 mM ADP, 5 mM phosphate, 5 mM glucose, 1.5 enzyme units hexokinase and 50 mM Tris-HCl pH 7.5. The reaction was stopped by adding $HClO_4$, final conc. 4%. After neutralisation with KOH-Tris to pH 7.6, glucose-6-phosphate or ATP was measured enzymatically with an Aminco–Chance double-beam spectrophotometer [7]. Acetylene reduction was measured according to Drozd and Postgate [8] in the presence of: 5 mM ATP, 2.5 mM $MgCl_2$, 50 mM phosphate pH 7.5 and 20 mM $Na_2S_2O_4$. Final vol 1 ml. The reaction was started by adding 4% acetylene. Protein was determined by the biuret method as modified by Cleland and Slater [9].

3. Results and discussion

It has been recently reported [10] that phosphotransacetylase activity is present in PDC while also the presence of acetate kinase could be demonstrated in Azotobacter. It thus appeared necessary to investigate the direct synthesis of ATP from pyruvate, ADP and phosphate in the presence of the appropriate cofactors:

Abbreviations: TPP: thiamine pyrophosphate; PDC: pyruvate dehydrogenase complex; DTT: dithiothreitol.

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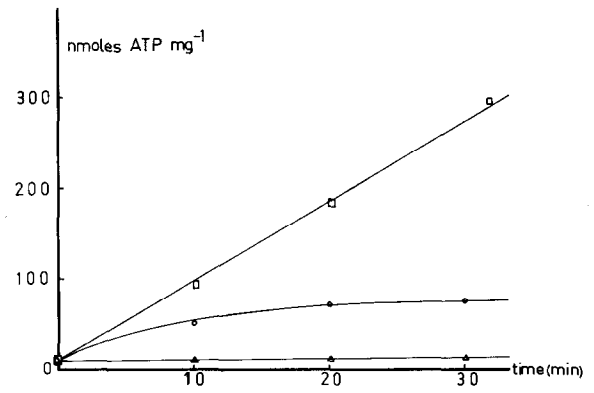

Fig. 1. Anaerobic ATP synthesis from pyruvate. The reaction was started by adding 8.6 mg crude extract to 2.0 ml standard reaction mixture as described under Methods. At the times indicated samples were taken and the reaction was stopped by addition of $HClO_4$ (final conc. 4%); (□–□–□): standard condition; (○–○–○): standard condition minus glucose and hexokinase; (△–△–△): standard condition minus pyruvate.

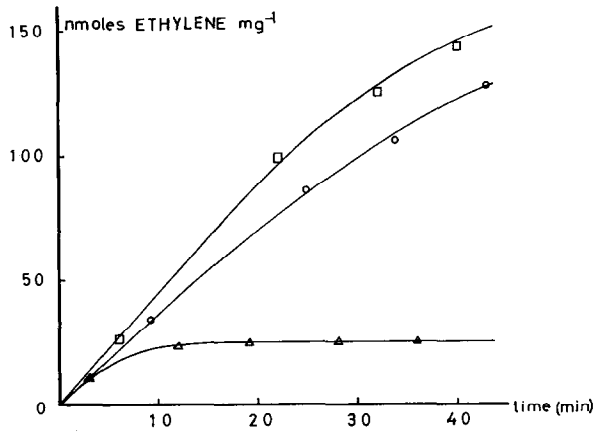

Fig. 2. Acetyl-phosphate as ATP regenerating system for acetylene reduction in a crude extract of Azotobacter. The reaction was started by the addition of acetylene to the acetylene reduction mixture, described under Methods. Per incubation 3.25 mg crude extract was used. At the points indicated gas samples were taken and analysed for ethylene; (□–□–□): acetylene reduction mixture plus 30 mM creatine phosphate and 0.25 mg creatine phosphokinase; (○–○–○): acetylene reduction mixture plus 15 mM acetyl phosphate; (△–△–△): acetylene reduction mixture.

TPP, CoA, NAD^+, apart from normal oxidative phosphorylation.

Fig. 1 shows that the ATP synthesis from pyruvate occurs; the process is linear over at least a 30 min period. Without an ADP regenerating system a rapid product inhibition is observed, probably due to acetyl CoA [10]. Thus it can be expected that intermediates like acetyl CoA and acetyl phosphate will be effective as energy donors in nitrogen fixation. As shown in fig. 2 acetyl phosphate in the presence of endogenous acetate kinase is very effective as an ATP regenerating system. Almost as effective as the classical ATP regenerating system for the nitrogenase, e.g. creatine phosphate with creatine phosphokinase.

The significance of this substrate-bound phosphorylation process for the energy supply in the Azotobacter is clearly illustrated in table 1. With pyruvate, under an atmospheric oxygen tension (pO_2 0.2), ATP is synthesized via the oxidative phosphorylation as well as by the substrate-bound phosphorylation. Without oxygen only the substrate-bound phosphorylation is observed. At low pO_2 (0.02), oxidative phosphorylation is lowered correspondingly but the amount of substrate-bound phosphorylation has not changed. The results show that substrate-bound phosphorylation under atmospheric oxygen tension is almost as effective as oxidative phosphorylation. Substrate-bound phosphorylation can be inhibited by the addition of oxaloacetate or malate plus oxygen (not shown) to the reaction mixture. Yates [11] reported anaerobic ATP synthesis in a crude extract of *Azotobacter chroococcum* and suggested that phosphate is esterified as a result of electron transfer to nitrogenase. According to our results these observations can be better explained as being due to the substrate-bound phosphorylation with endogenous pyruvate. Our results clearly indicate that pyruvate can serve as an energy source under anaerobic conditions while furthermore an intermediate of the reaction from pyruvate to ATP and acetate e.g. acetyl phosphate, can provide ATP for the nitrogen fixation. Thus pyruvate is the physiological energy donor for the anaerobic process of nitrogen fixation in Azotobacter. NADH formed during the PDC reactions can provide directly [11] or indirectly via NADPH [12] formed as the result of the pyridine nucleotide transhydrogenase reaction [13, 14] reducing equivalents for the nitrogen fixing system. Also in this respect pyruvate seems to play a similar role in *A. vinelandii* as described in Clostridium [3].

Table 1

ATP production by oxidative phosphorylation and substrate-bound phosphorylation under different oxygen tensions.

Conditions		pO_2		
		0.2	0.02	0.00
A	Standard	11.3	9.1	6.0
B	Standard minus pyruvate plus 20 mM NADH	6.8	3.6	1.5
C	Standard plus 40 mM oxaloacetate	–	–	1.5
D	Standard minus pyruvate	–	–	1.8
E	A–B: substrate-bound phosphorylation	4.5	5.5	4.5

The reaction mixture contained: standard reaction mixture (see Materials and methods) and 1.5 enzyme units/ml citrate synthase. ATP synthesis is expressed as nmoles$\cdot mg^{-1} \cdot min^{-1}$.

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